Angiogenesis in bone marrow of myelodysplastic syndrome patients

Krzysztof Mądry[1], Jadwiga Dwilewicz-Trojaczek[1], Wael Suleiman[2], Małgorzata Paszkowska-Kowalewska[1], Bogna Ziarkiewicz-Wróblewska[2]

[1] Department of Hematology, Oncology and Internal Medicine, Medical University, Warszawa, Poland

[2] Department and Chair of Pathomorphology, Medical University, Warszawa, Poland

Abstract: Introduction. Angiogenesis is an element of physiological and some pathological processes. Recently various investigators have reported that angiogenesis is associated not only with solid tumors but also with hematological malignancies. **Objectives.** The aim of this study was to assess angiogenesis in patients with myelodysplastic syndromes. **Patients and methods.** We have measured bone marrow microvessel density (MVD) in 43 myelodysplastic syndrome (MDS) patients and in 10 subjects of group control – 10 lymphoma patients (their bone marrow was free of disease). It was estimated by immunohistochemical method using anti-CD31 and anti-CD34 monoclonal antibodies. In 14 MDS patients and 6 healthy donors we also measured serum vascular endothelial growth factor (VEGF) by an immunoenzymatic method. **Results.** Higher MVD numbers were found in MDS patients when compared to control bone marrows. The highest number of MVD was in RAEBt and CMML MDS subtypes according to FAB. There was no correlation between MVD and biological features of MDS patients except for the age (negative correlation). Bone marrow microvessel density does not influence significantly the overall survival. The serum VEGF concentration in MDS patients was higher than in healthy donors, but the difference was not statistically significant. The VEGF levels did not correlate with MVD. **Conclusions.** We concluded that angogenesis is enhanced in bone marrow MDS patients though its mechanism is not yet fully understood.

Key words: angiogenesis, microvessel density, myelodysplastic syndromes, vascular endothelial growth factor

INTRODUCTION

Angiogenesis is the formation of new blood vessels from an existing vasculature. Angiogenesis has a major role in tumor growth, dissemination and metastasis in solid tumor [1,2]. The studies of early stage breast cancer, melanoma and prostate cancer suggested the angiogenesis to be a prognostic factor [3-5]. Moreover, it has been proposed that angiogenesis could play a cardinal role in myeloma [6] and lymphoma [7]. Interesting data has been drawn from investigations for angiogenesis in the acute myeloid leukemia (AML). Angiogenic factors might play the role not only in neovascularization, but also serve as autocrine and paracrine stimuli for blasts [8].

Aguayo et al. reported that the vascular endothelial growth factor (VEGF) concentration in bone marrow and in the peripheral blood is an independent prognostic factor in patients with the AML [9]. Karkolopoulou et al. reported reversal correlation between the total vascular area (TVA) in bone marrow and overall survival, and between microvessel density (MVD) and disease free survival in patients with the MDS [10]. It is interesting, but little is known about the association of angiogenesis with the subtypes of myelodysplastic syndrome (MDS) according to the FAB classification (French-American-British classification), progression to the AML and bone marrow cellularity.

Angiogenesis might be investigated using direct methods: quantitative and qualitative microvessels estimation in the study specimen, and using indirect measure methods to assess levels of angiogenic factors in serum, bone marrow or urine.

The aim of this study was to assess angiogenesis in patients with myelodysplastic syndromes by using measures of microvessel density (MVD) and the VEGF expression in serum and to define whether both methods – direct and indirect – correspond to each other. The second aim was to compare angiogenesis in the less aggressive subtypes MDS (RA, RARS) with the more aggressive (RAEB, RAEBt) and define angiogenesis relations with biological and clinical characteristics of the patients with MDS.

Correspondence to:
lek. Krzysztof Mądry, ul. Okrętowa 74a, 01-309 Warszawa, Poland, phone: 693-351-797, e-mail: kmadry@amwaw.edu.pl
Received: April 22, 2007. **Accepted in final form:** June 15, 2007.
Conflict of interest: none declared
Pol Arch Med Wewn. 2007; 117 (4): 145-150
Copyright by Medycyna Praktyczna, Kraków 2007

PATIENTS AND METHODS

Blood vessels in the bone marrow

We evaluated bone marrow biopsies (BMBs) from 43 consecutive patients with myelodysplastic syndrome diagnosed and treated in the Department of Hematology, Oncology and Internal Medicine at the University Hospital in Warsaw in the years 1998–2003. Bone marrow from 10 patients with Hodgkin's disease and non-Hodgkin's disease without the evidence of bone marrow involvement served as a control group. The MDS diagnosis was made according to the FAB classification by means of the BM aspirate stained with the May-Grunewald-Giemsa and trephine biopsy. The BM biopsy tissues were fixed in Oxford's solution and embedded in paraffin. The 0.4 μm- thick sections were stained with haematoxylin-eosin and the Gomori for the presence of reticulin fibre. The immunohistochemical analysis was made using the following monoclonal antibodies: CD31 IC70A and CD34 cl II QBEnd/10 (DakoCytomation, En Vision kit). The MVD enumeration was made following the estimation of all specimen (5–27 fields in trephine) evaluated at 40× magnification to the presence of endothelial cells. Next, the mean values of vessels per a single field in the trephine were counted.

Determination of VEGF in serum

The VEGF was measured in the serum of 14 patients with the MDS and in 6 healthy patients from the control group. Peripheral blood was collected in sterile tubes, centrifuged at 400g for 15 min. Then the plasma was stored in aliquots at –20°C. The level of VEGF was determined using a quantitative enzyme-linked immunosorbent assay (ELISA) technique (Quantikine; R and D Systems, Minneapolis, USA).

Statistical analysis

Statistical comparisons were performed using Spearman correlations. The p-value of <0.05 was considered statistically significant. Overall survival rates were performed using Kaplan-Meier plots.

RESULTS

Characteristics of the study group

Among 20 patients from the group of 43 patients with MDS, in whom the MVD was analyzed, the RA was diagnosed in 1 – RARS, in 4 – RAEBt, in 2 – CMML; the control group included 10 patients with lymphoma or non-Hodgin lymphoma without the evidence of bone marrow involvement.

Table 1. Patients characteristics

number of patients	43
mean age	66
gender	
male	26
female	17
FAB	
RA	20
RARS	1
RAEB	16
RAEBt	4
CMML	2
primary/secondary MDS	
primary	41
secondary	2
IPSS	
small	9
medium-1	21
medium-2	8
large	5
bone marrow cellularity	
hypercellularity	15
mediumcellularity	24
hypocellularity	4

CMML – chronic myelomonocytic leukemia, FAB – French-American-British classification, IPSS – international prognostic index, MDS – myelodysplastic syndrome, RA – refractory anemia, RAEB – refractory anemia with excess of blasts, RAEBt – refractory anemia with excess of blasts in transformation, RARS – refractory anemia with ring sideroblasts

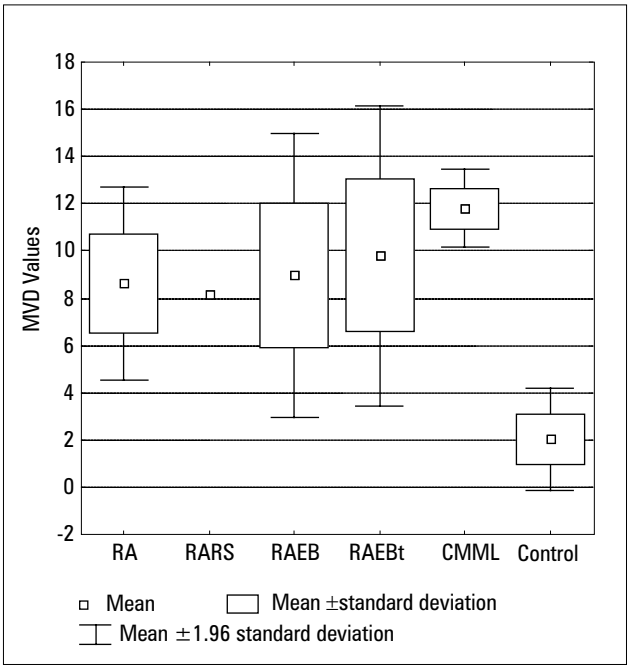

Fig. 1 MVD values in the different subtypes of MDS and in the control group. Abbreviations: MVD – microvessel density, others – see table 1

Table 2. MVD in different subtypes of MDS

Diagnosis	number of patients	MVD mean	MVD range	MVD median
controls	10	2.3	0.5–3.4	2.4
MDS	43	8.86	3.2–14.0	8.3
RA/RARS	21	8.1	4.3–12.1	8.2
RAEB	16	9.0	3.2–14.0	10.2
RAEBt	4	11.8	11.2–12.2	12.1
CMML	2	11.2	12.2–12.4	11.8

MVD – bone marrow (microvessel density). Others – see table 1

41 patients had the primary MDS, 2 patients had the secondary MDS. The median age of patients with the MDS was 69 years (range 23–87 years), including 18 women and 25 men. The patients were enrolled in the study based on the international prognostic scoring system (IPSS); 9 patients had small indexes (0 point), 21 – medium-small indexes (0.5–1 point), 8 – medium-large indexes (1.5–2 points), 5 – large indexes (≥2.5 points). Bone marrow hypercellularity was found in 15 patients, medium cellularity – in 24, hypocellularity – in 4 (tab 1). Cytogenetic investigations were carried out in 27 patients; among them: 20 patients had karyotype with good prognosis, 1 – with intermediate, and 6 – with bad prognosis.

Blood vessels in bone marrow

The mean microvessel density in the study group was estimated by the immunohistochemical method using anti-CD31 and anti-CD34 monoclonal antibodies. The MVD values in the MDS patients were significantly higher than in the control group and were 8.86 and 2.3 respectively. This difference was statistically significant. Among the patients with MDS the lowest MVD values were observed in the patients with RA/RARS – 8.1, the highest MVD values in the RAEBt patients – 11.8 (tab 2, fig. 1). No significant differences were found between the RA/RARS and RAREB/RAEBt subsets ($p > 0.05$). Significant differences were found between the RA/RARS and RAEBt subsets ($p < 0.05$) and also the RA/RARS and CMML ($p < 0.05$) (tab. 2). In the Spearman tests the MVD correlated reversal with age. The patients with hypocellular bone marrow were compared with the patients with medium-hypercellular bone marrow – hypocellular BM, the mean MVD 5.17 (median 4.3; min. 3.2, max 8.2) and medium – BM hypercellularity, the mean MVD 9.58 (median 9.03; min. 6, max. 14). The differences between these groups were not statistically significant. Among the patients with higher MVD (MVD >9) higher percentages of blasts in the BM (respectively: 9% and 6%), higher levels of vitamin B12 (respectively: 742 pg/ml and 477 pg/ml), higher erythropoietin concentrations in blood (respectively: 192 U/l and 86 U/l) were observed than in other patients (MVD <9). The other parameters- haemoglobin, platelets, white blood cells, erythrocytes, haematocrit, iron, sedimentation rates, the LDH level and the IPSS were comparative in both groups. The VEGF concentration in the serum of the patients with higher MVD was slightly lower, the mean 275 pg/ml, in others 298 pg/ml ($p > 0.05$). Overall survival rates in both groups were similar, in the patients with higher MVD – 16.5 months, in the patients with lower MVD – 14 months (tab. 3).

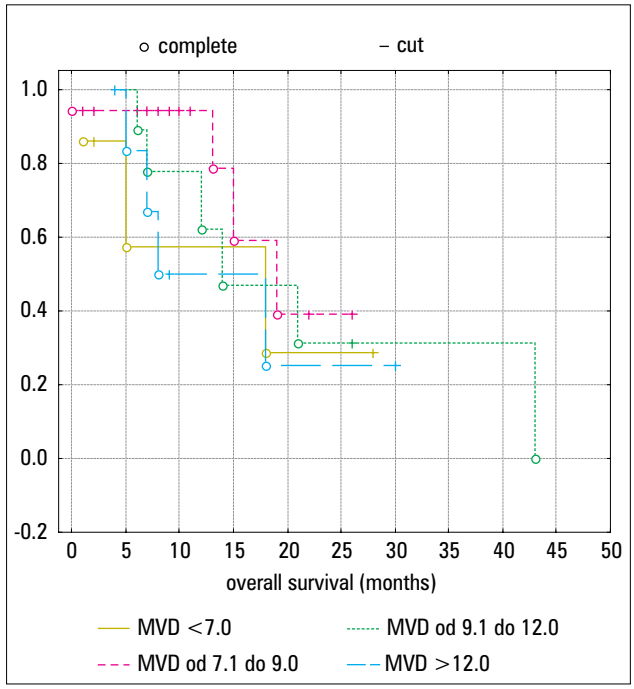

Fig 2. Overall survival determined by the MVD

Overall survival was analyzed in 4 ranges based on the values of MVD: group I – up to 7, group II – 7.1-9.0, group III – 9.1–12.0 and group IV – MVD >12.0. Overall survival did not differ in the study groups and was respectively: 12 months, 16 months, 16.1 months and 15 months (tab. 4) Kaplan-Meier curves show that the curves of patients with the lower BM MDV lie above the curves of patients with the higher MVD, which means that the overall survival of these patients (with lower MVD) is longer. The exception from this rule is the MVD <7.0 range, which curves lie below the higher MVD 7–12.0 (fig.2).

Table 3. Parameters determined by the MVD

Study parameter	MVD ≤9.0	MVD <9.0
total no. of patients	19	24
FAB RA/RARS	8	13
RAEB	7	9
RAEBt	2	2
CMML	2	0
IPSS		
small	6	3
medium-1	9	11
medium-2	4	5
large	1	4
gender		
male	13	2
female	6	12
age	66.9	67.2
cytogenetics – prognosis		
good	11	9
intermediate	0	1
bad	3	3
haemaglobin (g/dl)	8.22	8.72
RBC (T/l)	2.56	2.77
Hct (%)	24.6	25.7
platelets (G/l)	92.9	97.5
white blood cells (G/l)	6.01	4.0
blasts (%)	9.02	6.4
rate of sedimentation (mm/h)	67.4	61.7
VEGF (pg/ml)	275	298
LDH (U/l)	616.9	629
erythropoietin (IU/l)	192	86
iron (μg/dl)	124.1	147
vitamin B_{12} (pg/ml)	742	477
RBC transfusion (Unit/month)	3.68	2.1
death/alive		
death	11	7
alive	8	17
overall survival (days)	495	441

VEGF – vascular endothelial growth factor, others – see table 1. cytogenetics – prognosis: good if del 5, del 20 or – Y; bad, if chromosome 7 abnormalities or at least 3 abnormalities; intermediate, if no abnormalities or others than above

VEGF in serum

The VEGF concentration was measured in plasma samples obtained from14 patients with the diagnosis of MDS including 8 patients with the RA type, 3 patients with the RAEB type and 2 patients with the CMML (tab.5). In all patients the bone marrow MVD was also performed. The control group included six healthy volunteers. In the control group the mean VEGF concentration in plasma was 186.8 pg/ ml, the median was 179 pg/ ml (range, 33–392), in the patients with MDS

Table 4. Overall survival in ranges according to the MVD values

Range of MVD values	Mean OS (months)	Median OS (months)	Ranges of values OS (months)
≤7.0	12.0	9.2	1–33
7.1–9.0	16.0	14.2	0.9–33.9
9.1–12.0	16.1	12.3	1–43.3
>12.0	15.0	13.0	5.4–36.9

OS – overall survival, others – see tab 1

267.7 pg/ ml, the median 152 (range 12.5–810). This difference was not statistically significant because of a large mutability of results in both groups and a small number of patients.

No correlation between the VEGF concentration in serum and biological parameters in the patients with MDS was found. The exception was the correlation with the LDH levels. There was no correlation between the VEGF in serum with the bone marrow MVD.

DISCUSSION

In the current study we documented the increased bone marrow angiogenesis in patients with the MDS; consistent with the data of Pruneri et al., the highest MVD values were observed in the most aggressive subtypes MDS RAEBt and CMML [12]. In accordance with previous reports, the mean MVD values in patients with the MDS were higher than in the control group and lower than in patients with the AML [11,12]. In other subtypes RA, RARS and RAEB, the results differed slightly. These data suggested that increased angiogenesis might be associated with readiness to progression to leukemia. It is difficult to establish the relation between angiogenesis and progression to leukemia. Bellamy et al. reported the VEGF-Flt-1 and Flk-1 receptors expression in blasts [8]. Hussong et al. demonstrated, using the PCR, the VEGF expression in cell lines from patients with the AML [13]. Endothelial cell mitogenic factors (first of all VEGF) are the potent mitogens not only for endothelial cells, but also blasts, and can inhibit apoptosis [14]. Our findings indicate that the patients with the increased MVD had lower haemoglobin levels, higher erythropoietin in blood and lower requirement for blood transfusion compared with other patients. It might suggest that increased angiogenesis is secondary to hypoxia.

We did not find that the MVD was a prognostic factor in the MDS. The differences between overall survival rates in patients with the different MVD were not statistically significant. Karkolopoulou et al. reported that the MVD correlated with the risk of progression to leukemia and the overall survival correlated with the length of lower axis bone marrow vessels, which the longer, the longer overall survival [10]. Mentioned authors suggested that, in the RA and RARS subtypes, the diameter of vessels is larger than in other subtypes of MDS.

Table 5. VEGF concentration in serum and the MVD among the patients with MDS and in the control group

Study group	Type of MDS according to FAB	MVD	VEGF (pg/ml)
1.	RA	12.1	678
2.	RA	7.2	456
3.	RA	8.3	97
4.	RA	9.1	157
5.	RA	8.1	810
6.	RA	8.3	139
7.	RA	9.0	520
8.	RA	8.2	38
9.	RAEB	11.3	149
10.	RAEB	12.5	147
11.	RAEB	8.1	209
12.	RAEB	8.7	603
13.	CMML	11.2	133
14.	CMML	12.4	28
1.	control	–	33
2.	control	–	301
3.	control	–	222
4.	control	–	128
5.	control	–	392
6.	control	–	68

Abbreviations – see tables 1 and 2

We found a significantly higher bone marrow microvessel density in patients with bone marrow hypercellularity, but differences were not significant. This finding is in accordance with other authors [11,12]. It seems unlikely that the increased MVD was solely due to hypercellularity. The MVD was significantly lower in the AML than in the control group, in spite of the BM hypercellularity [12]. In our study there was no correlation between the VEGF concentration in serum and the MVD. This findings suggests that angiogenesis is induced by local factors and only in a small percentage achieves circulation. Also, it can be due to the complexity of angiogenesis process which is determined by various angiogenic factors and receptors, and, at last, because the VEGF can be bound by other cells than endothelial cells including megakaryocytes and platelets. The study group (14 patients) was too small to draw final conclusions. Aguayo et al. reported the elevated levels of VEGF in the serum of patients with the MDS and AML; the VEGF was an independent prognostic factor in patients with the AML [9]. On the other hand, Zorat et al. documented that there was no difference in the VEGF concentration between the patients with MDS and the control group [15]. It seems that the investigation of VEGF and its receptors in supernatants of cultured bone marrow cells may have higher value.

ACKNOWLEDGMENTS

The Authors would like to thank Prof. Wiesław Wiktor Jędrzejczak for critical reading of the manuscript and technical support.

REFERENCES

1. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971; 285: 1182-1187.
2. Ellis LM, Fiedler IJ. Angiogenesis and metastasis. Eur J Cancer. 1996; 32A: 2451-2455.
3. Gasparini G. Clinical significance of the determination of angiogenesis in human breast cancer: update of the biological background and overview of the Vicenza studies. Eur J Cancer. 1996; 32A: 2458.
4. Graham CH, Rivers J, Kerbel RS, et al. Extent of vascularisation as a prognostic indicator in thin (<0,076 mm) malignant melanoma. Am J Pathol. 1994; 145: 510.
5. Weidner N, Carroll PR, Flax J, et al. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Patol. 1993; 143: 401.
6. Vacca A, Di Loreto M, Ribatti D, et al. Bone marrow of patients with active multiple myeloma. Angiogenesis and plasma cell adhesion molecules LFA-1,VLA-4, LAM-1 and CD44. Am J Hematol. 1995; 50: 9-14.
7. Salven P, Teerenhovi L, Joensuu H. A high pretreatment serum vascular endothelial growth factor concetration is associated with poor outcome in non-Hodgin's lymphoma. Blood. 1997; 90: 3167-3172.
8. Bellamy WT, Richter L, Sirjani D, et al. Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. Blood. 2001; 97: 1427-1434.
9. Aguayo A, Kantarjian HM, Estey EH, et al. Plasma vascular endothelial growth factor levels have prognostic significance in patients with acute myeloid leukemia but not in patients with myelodysplastic syndromes. Cancer. 2002; 95: 1923-1930.
10. Korkolopoulou P, Apostolidou E, et al. Prognostic evaluation of the microvascular network in myelodysplastic syndromes. Leukemia. 2001;15: 1369-1376.
11. Aguayo A, Kantarjian H, Manshouri T, et al. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood. 2000; 96: 2240-2245.
12. Pruneri G, Bertolini F, Soligo D, et al. Angiogenesis in myelodysplastic syndromes. Br J Cancer. 1999; 8: 1398-1401.
13. Hussong JW, Rodgers GM, Shami PJ. Evidence of increased angiogenesis in patients with acute myeloid leukemia. Blood. 2000; 95: 309-313.
14. Smolich BD, Yuem HE, West K, et al. The antiangiogenic protein kinase inhibitors SU5416 and SU 6668 inhibit the SCF receptor (c-kit) in human myeloid leukemia cell line and in acute leukemia blasts. Blood. 2001; 97: 1413-1421.
15. Zorat F, Shetty V, Dutt D, et al. The clinical and biological effects of thalidomide in patients with myelodysplastic syndromes. Br J Hematol. 2001; 115: 881-894.